Effect of nitric oxide synthase inhibitor (L-NAME) on substance P-induced vasodilatation in the dental pulp

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Abstract

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Aim To investigate the vasodilator mechanisms of pulpal vessels, especially the involvement of nitric oxide (NO), during pulpal inflammation.

Methodology Eleven cats were prepared for intra-arterial administration of test agents through a lingual artery. The pulpal blood flow was measured by laser Doppler flowmetry from ipsilateral mandibular canine teeth. By using the NO synthase (NOS) inhibitor N^G-nitro L-arginine methyl ester (L-NAME), the effects of L-NAME on various vasodilators, such as Substance P (SP)-, calcitonin-gene related peptide (CGRP)-, and papaverine-induced vasodilatation, were compared *in vivo* in 11 feline dental pulps.

Results L-NAME pretreatment potentiates SP-induced vasodilatation for a duration of approximately 5 h. The increase of pulpal blood flow ranged from 91.47 to 109.91%, which was significantly different from SP injection alone (48.79%, $P < 0.05$). Other vasodilators such as CGRP and papaverine did not respond to L-NAME pretreatment.

Conclusions This study demonstrates that NOS inhibitor L-NAME administration alone has insignificant effects on pulpal blood flow, although L-NAME pretreatment can potentiate SP-induced vasodilatation, probably via increased activity in the enzyme guanylate cyclase. CGRP and papaverine did not respond to L-NAME pretreatment, indicating that they are not mediated via an endothelium-dependent mechanism.

Keywords: cGMP, CGRP, endothelium-dependent vasodilatation, L-NAME, nitric oxide, Substance P.

Introduction

Vascular relaxation is an important parameter in regulating homeostasis of physiological functions. In dental pulp, tissue damage releases inflammatory mediators such as prostaglandins and bradykinins. These inflammatory mediators have effects on nerves and blood vessels that result in nerve hypersensitivity, vasodilatation and vascular leakage. Excitation of C-fibres can trigger the release of neuropeptides such as Substance P (SP) and calcitonin-gene related peptide (CGRP). SP is known as a very potent vasodilator in the pulpal microvasculature. These reactions can increase pulpal tissue pressure and persistent pulpal vasodilatation (Kim 1990). Furchgott & Zawadzki (1980) demonstrated that the vascular relaxation effect is mediated by a liable humoral factor released from endothelial cells and diffuses to the smooth muscle layer, known as endothelium-derived relaxing factor (EDRF). Considering the similarities in pharmacological behaviour, chemical nature and biological function, many researchers suggested that EDRF might be nitric oxide (NO; Palmer *et al.* 1987, Furchgott 1988, Ignarro *et al.* 1988).

NO is a free radical gas with a half-life of only 3–5 s in oxygenated physiological salt solution (Palmer *et al.*

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1987). The amino acid L-arginine is the precursor for the synthesis of NO by vascular endothelial cells. The enzyme that synthesizes NO from L-arginine is a mono- or dioxygenase enzyme system termed NO synthase (NOS). All NOS use free L-arginine and molecular oxygen as substrates to produce NO and L-citrulline. L-citrulline is then shunted into a metabolic pathway that regenerates free L-arginine (Meller & Gebhart 1993); this is the L-arginine: NO pathway.

Numerous analogues of L-arginine with modification at the terminal guanidino nitrogen and/or the carboxyl terminus are widely used to inhibit the production of NO. Examples of these compounds are: N^G-nitro L-arginine (L-NA) and its methyl ester analogue N^G-nitro L-arginine methyl ester (L-NAME), N^G-amino L-arginine (L-NAA) and N^G-monomethyl L-arginine (L-NMMA). They inhibit the release of NO by competitive inhibition of NOS (Lambert *et al.* 1991).

In the vascular system, NOS is expressed constitutively in the endothelial cells. Both shear stress and deformation of vascular endothelium, which accompany pulsatile flow through blood vessels, stimulate NO release through poorly defined mechanisms (Moncada & Higgs 1991). As a result of its small molecular size and high lipophilicity, NO can readily enter into nearby smooth muscle cells to activate cytosolic guanylate cyclase. Activated guanylate cyclase then converts guanosine triphosphate (GTP) into cyclic guanosine monophosphate (cyclic GMP or cGMP). cGMP may elicit muscle relaxation through influences on the Na^+–Ca^{2+} exchange, or activates a specific protein kinase, which results in the phosphorylation and inactivation of myosin light chain kinase. Inactivation of myosin kinase results in dephosphorylation of the myosin light chain and smooth muscle relaxation (Dinerman *et al.* 1993).

Previous studies suggested that NO is responsible for maintaining a physiological vasodilator tone in pulpal blood vessels (Kerezoudis *et al.* 1993, Lohinai *et al.* 1995, Berggreen & Heyeraas 1999). Its role in the pulpal microvasculature has also been studied in ferrets, and PBF results show that L-NAME administration causes a significant decrease in pulpal blood flow. (Berggreen & Heyeraas 1999). However, uncertainty remains regarding its role in pulpal neurogenic inflammation. Therefore, the purposes of this study were to investigate the role of NO in pulpal neurogenic inflammation by analysing SP-induced vasodilatation using L-NAME, and to compare the effect of L-NAME with other vasodilators such as CGRP and papaverine in the feline dental pulp.

Materials and methods

Animal preparation

The experiments were made on 11 cats of both sexes. Their ages ranged from 9 to 12 months, and their weight varied from 3 to 5 kg. Intra-peritoneal injection of sodium pentobarbital (Nembutal®, Abbott Laboratory, N. Chicago, IL, USA) was first administered (35 mg kg^{-1} of body weight) to induce anaesthesia. The femoral vein was cannulated for supplemental intravenous injections of sodium pentobarbital (2 mg kg^{-1} of body weight) to maintain anaesthesia. The femoral artery was also cannulated and connected to a Gould P23 physiological pressure transducer, which then linked to a Gould 2400S recorder (Gould Inc., Recording System Division, Cleveland, Ohio, USA). This allowed the continuous monitoring of systemic blood pressure. Both cannulae were flushed with heparin saline every 2–3 h to maintain patency. A lingual artery was isolated from the hypoglossal nerve bundle and was cannulated in a retrograde direction towards the junction of the common carotid artery. The tip of the cannula was positioned precisely at the junction with the bevel facing coronally to minimize blood-flow turbulence during drug administration. Ethical approval was sought and provided for study by the local institutional animal care and use committee.

Periapical radiographs of the mandibular canines were taken to ensure that the apices were mature and the pulpal canals were normal. The mandible was immobilized with dental stone and a steel rod that was anchored to a magnetic stand. The mandibular canine on the ipsilateral side of the cannulated lingual artery was prepared for measurement of pulpal blood flow by laser Doppler flowmetry. During preparation, the buccal enamel on the cervical third of the tooth was removed to expose an even surface of dentine with a diameter of 2 mm. Throughout the experiment, the surface was moistened with isotonic saline to prevent drying of the dentine.

A minimum of 45 min was required after cavity preparation for pulpal blood flow to return to the control level (Liu *et al.* 1987). Hence, all pulpal blood flow measurements were made after at least 60 min had elapsed from the time of the cavity preparation. A laser Doppler flowmeter (Periflux PF 3, Perimed, Stockholm, Sweden) was used to obtain pulpal blood flow in millivolt (mV) units. At the end of the experiment, the cat was euthanized with an intra-venous (i.v.) overdose of sodium pentobarbital.

Drug preparation and administration

Four substances were prepared for the study of pulpal blood flow, namely L-NAME, SP, CGRP and papaverine (Sigma Chem. Co., Saint Louis, MO, USA). These drugs were injected intra-arterially (i.a.) through the lingual artery cannula and were followed by 2–3 mL heparin-saline flush to ensure that none of the test substances were left in the cannula. Each drug, except L-NAME, was titrated at different concentrations to find out the optimal dose. At this optimal concentration, the drugs would have maximal local effect on the pulpal blood flow without significant systemic effect on arterial blood pressure. L-NAME at a concentration of 0.5 mg kg^{-1} was slowly injected through the lingual artery in all experimental animals. After L-NAME administration, test drugs were injected through the lingual artery every hour for 5–6 h.

Experimental procedure

Among the 11 cats, 6 cats were used to study the effect of L-NAME on SP-induced vasodilatation. Three cats were used for CGRP-induced vasodilatation, and two cats for papaverine-induced vasodilatation. The procedures were carried out, and all of the following substances were introduced via the lingual artery:

1 SP, CGRP or papaverine was injected alone to titrate the optimal concentration for the individual animal.

2 L-NAME 0.5 mg kg^{-1} was injected alone.

3 SP at doses of 0.004–0.04 μg kg^{-1} was injected every hour after L-NAME administration for 5–6 h.

4 CGRP at concentrations of 0.03–0.06 μg kg^{-1} was injected every hour after L-NAME administration for 5–6 h.

5 Papaverine was injected at doses of 0.2–0.5 mg kg^{-1} every hour after L-NAME administration for 5–6 h.

Data acquisition

Before each injection, at least 10–15 s of stable blood flow recordings served as baseline values. After drug administration, the reading on the laser Doppler flowmeter with the largest deviation from the base line value was taken as the experimental data. The pulpal blood flow, together with systemic blood pressure, was recorded continuously with a Gould 2400S direct writing recorder.

The experimental data were converted and expressed as a percentage change from the baseline value.

Results

Effect of intra-arterial injection of L-NAME, SP, CGRP and papaverine on pulpal blood flow

In reporting the results, each mean value was accompanied by its standard error of the mean (SEM), i.e. mean ± SEM, and the analysis of variance (ANOVA) and paired *t*-test were used for statistical analysis. A *P*-value less than 0.05 was considered statistically significant (Figs 2 and 3).

The intra-arterial injection of L-NAME at 0.5 mg kg^{-1} caused an average increase of 16.38 ± 6.34% in pulpal blood flow, before returning to baseline within 5 min. Both systemic arterial blood pressure and pulpal blood flow remained stable during L-NAME administration (Figs 1 and 2).

The intra-arterial injection of exogenous SP at doses of 0.004–0.04 μg kg^{-1} resulted in increases of pulpal blood flow with an average value of 48.79 ± 11.72%. This increase in pulpal blood flow was dose dependent and occurred about 15 s after the injection, reaching its peak effect in another 20 s. The blood flow gradually returned to its baseline value after approximately 3 min. SP injection in this concentration range caused very minimal systemic blood pressure changes (Figs 1 and 2).

Injection of CGRP through the lingual artery at concentrations of 0.03–0.06 μg kg^{-1} caused an average increase of 36.98 ± 7.53% in pulpal blood flow. Increase in pulpal blood flow occurred about 15 s after the injection and reached a maximum level after 60 s. It took approximately 6 min for the blood flow to return to baseline value. There was no significant change in systemic blood pressure after CGRP administration (Figs 1 and 2).

Papaverine at concentrations of 0.1–0.5 mg kg^{-1} injection into the lingual artery resulted in an average increase of pulpal blood of 24.85 ± 5.59%. Papaverine also caused a slight, transient decrease in systemic arterial blood pressure in 60% of the cases. However, this change was statistically insignificant and soon returned to baseline levels (Figs 1 and 2).

Effect of L-NAME on SP-induced vasodilatation of pulpal blood vessels

Intra-arterial injection of SP after L-NAME administration produced a notable increase in pulpal blood flow. In the first hour after L-NAME injection, SP resulted in an increase of pulpal blood flow of 93.74 ± 12.46%, which was a significant difference from SP alone (48.79 ± 11.72%; *P* < 0.05).
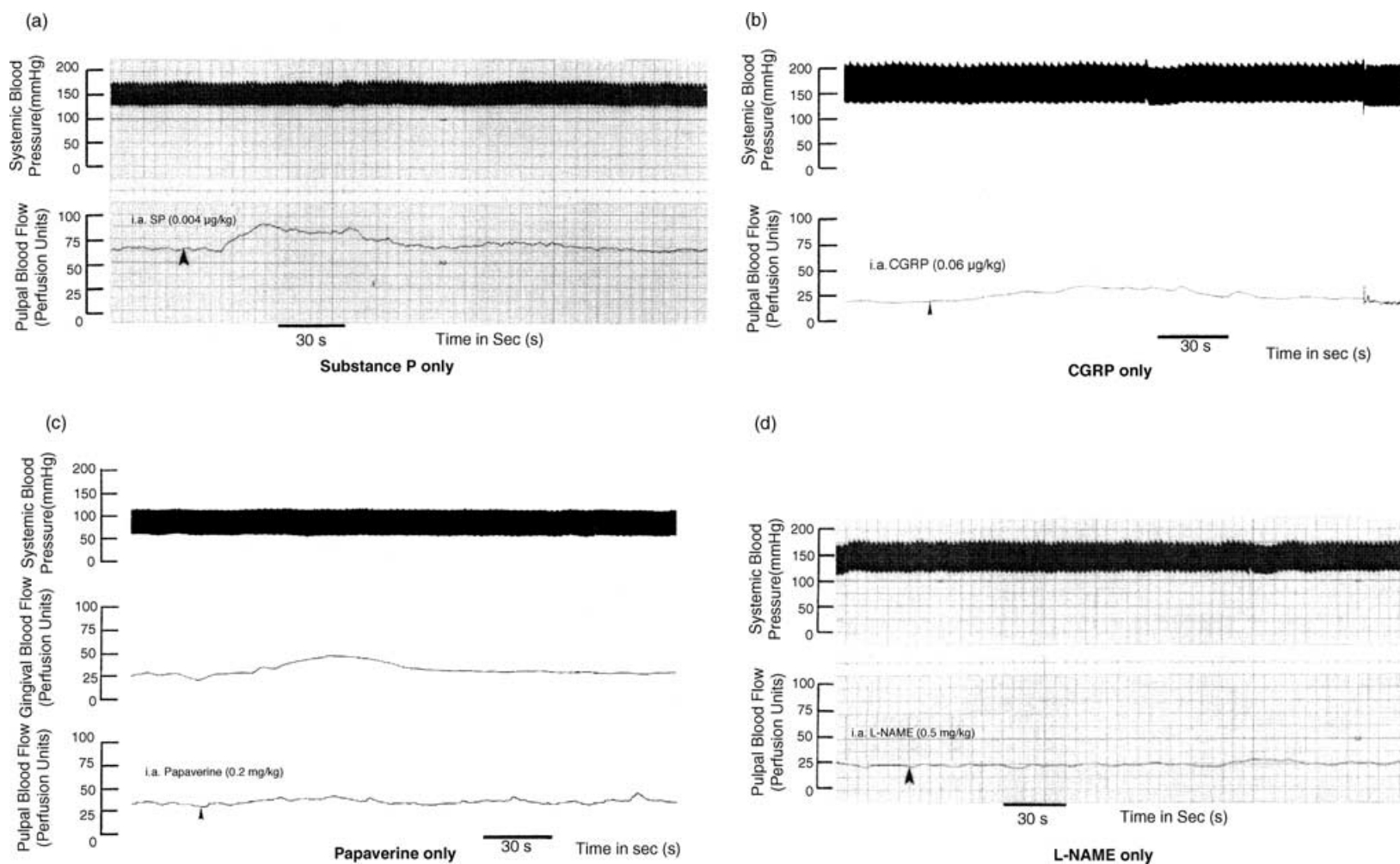
Figure 1 Original polygraph simultaneous recordings of the systemic blood pressure and pulpal blood flow when intra-arterial injection of (a) L-NAME (0.5 mg kg^{-1}), (b) SP (0.004–0.04 g kg^{-1}), (c) CGRP (0.03–0.06 g kg^{-1}) and (d) papaverine (0.1–0.5 mg kg^{-1}).
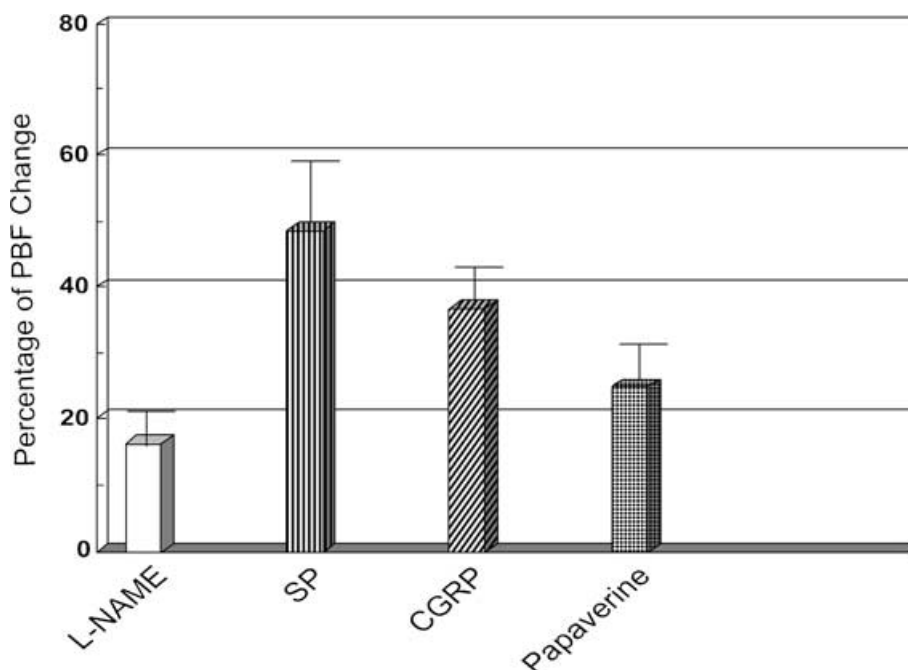
Figure 2 Bar diagram illustrating the effect of intra-arterial injection of L-NAME, SP ($n = 6$), CGRP ($n = 3$), and papaverine ($n = 2$) on pulpal blood flow. The results are expressed as mean $\pm$ SEM.

Substance P injection 2 h after L-NAME administration, caused a pulpal blood flow increase by $109.91 \pm 16.94\%$ from the baseline value ($P < 0.05$). The SP injection increased the pulpal blood flow by 91.47 ± 14.66, 98.60 ± 20.55 and $92.70 \pm 20.43\%$ at 3, 4 and 5 h, respectively, after L-NAME administration. Each blood flow increase was significant in response to SP alone ($P < 0.05$). However, at the sixth hour after L-NAME administration, SP only increased pulpal blood flow by 31.9% ($P > 0.05$) (Fig. 3).

Effect of L-NAME on CGRP-induced vasodilatation of pulpal blood vessels

Intra-arterial injection of CGRP every hour after L-NAME administration caused increases in pulpal blood flow. The average blood flow increases during the 5-h period: $43.62 \pm 12.45\%$ in the first hour; $36.95 \pm 6.85\%$ in the second hour; $23.75 \pm 2.87\%$ in the third hour; $41.39 \pm 11.44\%$ in the fourth hour; and $31.09 \pm 6.62\%$ in the fifth hour. There was no statistical significance
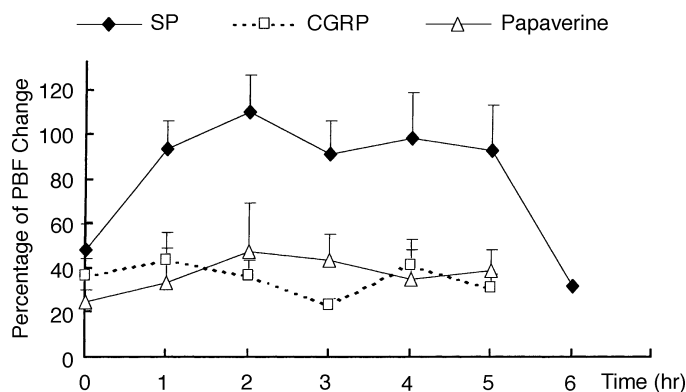
Figure 3 Comparison of the effect of L-NAME pretreatment on SP. The potentiation effect of L-NAME on SP-induced vasodilatation after the first and up to 5 h is significant ($P < 0.05$). Results are expressed as mean $\pm$ SEM.

in these data and on the effect of CGRP administration alone ($36.98 \pm 7.53\%$; $P > 0.05$) (Fig. 3).

Effect of L-NAME on papaverine-induced vasodilatation of pulpal blood vessels

Papaverine intra-arterial injection following L-NAME administration also increased pulpal blood flow. In the first hour, papaverine increased pulpal blood flow by an average of $33.83 \pm 16.49\%$ ($P > 0.05$). This was followed by a $47.79 \pm 21.46\%$ increase in the second hour ($P > 0.05$), a $43.45 \pm 12.47\%$ increase in the third hour, a $35.47 \pm 12.81\%$ increase in the fourth hour and a $38.72 \pm 9.42\%$ increase in the fifth hour. The increases in pulpal blood flow from papaverine injection alone ($24.85 \pm 5.59\%$; $P > 0.05$) during different time periods were not significant (Fig. 3).

Discussion

Numerous recent studies suggest that NO plays an important role in both physiological and pathological effects on various organ systems (Furchgott 1988, Moncada & Higgs 1991, Dinerman *et al.* 1993, Kerezoudis *et al.* 1993, Stone *et al.* 1995). As a result of the instability and short half-life of NO, it is very difficult to study and monitor the NO effects on the target tissue by direct observation. Therefore, researchers use indirect approaches, such as false substrates, to determine the involvement of NO in a specific function. Many congeners of arginine that block the action of NOS have been described. L-NMMA was the first L-arginine analogue shown to inhibit NO formation from L-arginine in a stereo-specific manner (Moncada *et al.* 1989). However, its low potency and lack of selectivity among different tissues limit L-NMMA to *in vitro* experiments. L-NA, the simplest form of L-arginine analogue, is an inhibitor of NO both *in vivo* and *in vitro*. Its methyl ester analogue L-NAME is more lipophilic and more selective; therefore, L-NAME is very useful for *in vivo* experiments (Buxton *et al.* 1993).

In a preliminary study, L-NAME intravenous injection at a higher concentration ($25 \, \text{mg kg}^{-1}$) could induce long-lasting increases in systemic arterial blood pressure for approximately 4 h. However, intra-arterial infusion of L-NAME at a lower concentration ($0.1 \, \text{mg kg}^{-1}$ h over a 7-h period or $0.5 \, \text{mg kg}^{-1}$ slow injection) did not change the systemic blood pressure. To eliminate the possible influence of systemic blood pressure changes on the local pulpal blood flow, the dose of L-NAME used in the present study was much lower than that used in

other studies (Kerezoudis *et al.* 1993, Lohinai *et al.* 1995, Berggreen & Heyeraas 1999). Using a low L-NAME concentration may also contribute to the absence of permanent systemic blood pressure change after prolonged infusion.

In the process of investigating SP, L-NAME and other NO antagonists have played an important part in establishing the role of SP in endothelium-dependent vasodilatation. Numerous studies have reported that L-NAME attenuates SP-induced vasodilatation in different preparations. These include porcine splenic artery (Lot & Wilson 1994), rabbit hindlimb skin (Gustafsson *et al.* 1994), cat pulmonary vascular bed (Cheng *et al.* 1994), guinea-pig coronary bed (Vials & Burnstock 1992), as well as isolated human ovarian vein (Stone *et al.* 1995) and pial artery (Petersson *et al.* 1995). Considering the findings of these studies, the mechanism of SP-induced vasodilatation appears to be the result of increasing endogenous NO production (Gustafsson *et al.* 1994).

Other studies have reported that L-NAME failed to reduce antidromic vasodilatation induced by SP and CGRP in the rat hindpaw skin (Holzer & Jocic 1994), dog oesophageal mucosal blood flow (Sandler *et al.* 1993) and ferret pulpal blood flow and tissue pressure (Berggreen & Heyeraas 1999). These findings suggest that SP and CGRP do not induce vasodilatation through release of NO (Holzer & Jocic 1994, Berggreen & Heyeraas 1999). Berggreen & Heyeraas (1999) conducted an elegant study to measure the pulpal interstitial pressure and blood flow of ferret canine teeth, simultaneously. Neurogenic inflammation was induced by electric stimulation of the inferior alveolar nerve, which in turn caused release of both SP and CGRP from the sensory nerve terminals. Their results revealed that L-NAME administration caused a significant rise in interstitial fluid pressure and systemic arterial pressure, L-NAME had no effect on the vasodilatation induced by electric stimulation. These divergent interpretations might be explained by the different species used, by the different methodologies of tooth preparation, by the different concentrations of L-NAME administered and by the different means of neurogenic inflammation induction.

The present study demonstrates that L-NAME has a potentiation effect on SP-induced vasodilatation in the feline dental pulp. Kerezoudis *et al.* (1993) reported similar findings in rat dental pulp and oral mucosa. Considering the results that L-NAME potentiates SP-induced vasodilatation, two possible mechanisms are proposed:
1 L-NAME produces preconstriction of pulpal blood vessel that enhances SP-induced vasodilatation.

2 Decreases in NO concentration by L-NAME increases the activity of soluble guanylate cyclase that then potentiates the effect of vasodilatory agents.

The resting tone of the pulpal vasculature is a mildly dilated state (Heyeraas Tønder & Naess 1978). Basal release of NO may play a role in maintaining the pulp vasculature in such a dilated state (Lohinai *et al.* 1995). There is indirect evidence that NOSs exist in the endothelial cells of pulpal vessel and sub-odontoblastic cell layers, as well as in odontoblasts (Kerezoudis *et al.* 1993). These constitutive NOSs produce endogenous NO that may be involved in the control of the pulpal vascular tone. L-NAME administration inhibits release of basal NO, and creates a preconstriction stage in pulpal vessels. Preconstriction shortens the distance between the A band and Z line of a sarcomere, thus allowing more sliding distance between actin and myosin during relaxation. If this hypothesis is correct, then L-NAME should be able to potentiate the effect of many vasodilators in addition to SP. Another hypothesis is that giving L-NAME decreases NO production in the endothelium, which in turn reduces the concentration of cGMP. As a result of negative feedback, decreased cGMP level will increase the activity of guanylate cyclase, the enzyme that converts GTP to cGMP. The introduction of any NO-dependent vasodilators will produce more cGMP in the cell because of superexcitation of guanylate cyclase, thus resulting in greater vasodilatation.

Whether the CGRP vasodilatory effects are mediated by release of EDRF or not remains controversial. In the present study, CGRP administrations increased pulpal blood flow by an average of 36.98%. There was no significant change of pulpal blood flow between CGRP alone and L-NAME pretreatment groups, indicating pulpal vasodilatation induced by CGRP may be NO-independent. Nonetheless, this also needs to be confirmed by future study of cGMP levels after CGRP administration.

The primary actions of papaverine are exerted on cardiac and smooth muscle. It acts directly on the heart muscle to depress conduction and prolong the refractory period. The most characteristic effect of papaverine is relaxation of the tonus of all smooth muscles. In the dental pulp microvasculature, papaverine infusion caused an increase in pulpal blood flow and a decrease in pulpal vascular resistance. In the present study, papaverine injections through the lingual artery increased an average of 24.85% of pulpal blood flow. There was no significant change of blood flow even after L-NAME pretreatment. Therefore, the vasodilatation effect of papaverine was through an endothelium-independent mechanism.

Previously the possible mechanisms for L-NAME to enhance the effect of SP-induced vasodilatation were discussed. The first one is that L-NAME administration inhibits the basal release of NO and creates a preconstriction state in the pulpal vasculature. Preconstriction may allow more sliding distance between actin and myosin during relaxation, and L-NAME should be able to potentiate the vasodilatory effect of SP and other vasodilators as well. However, the results from CGRP and papaverine disagreed with such hypothesis, as L-NAME pretreatment was unable to enhance the CGRP and papaverine effect, especially papaverine, which functioned as a direct smooth muscle relaxant. If preconstriction was the mechanism for enhanced vasodilatation after L-NAME administration, then maximal relaxation following papaverine treatment should be observable. The present results direct future experiments towards the other hypothesis, that is L-NAME decreases NO concentration, which in turn increases the activity of soluble guanylate cyclase and then potentiates the vasodilatation effect. By monitoring the cGMP level after L-NAME treatment, different vasodilator administrations and combination of both, the vasodilatation mechanism of pulpal inflammatory mediators such as SP and CGRP can be investigated in more detail.

Conclusion

This study has demonstrated that NOS inhibitor L-NAME administration alone has insignificant effects on pulpal blood flow. However, L-NAME pretreatment can potentiate SP-induced vasodilatation, probably through increasing the enzyme guanylate cyclase activity mechanism. The duration of the potentiation effect is approximately 5 h. Other vasodilators such as CGRP and papaverine did not respond to L-NAME pretreatment, indicating that they might be mediated in an endothelium-independent manner. Future experiments will be conducted to measure cGMP level of the dental pulp by radioimmunoassay (RIA) to investigate the cellular mechanism of SP- and CGRP-induced vasodilatation.

Acknowledgements

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